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tail (*Sd*) Mice in Somites That Form Following Notochord Degeneration

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***Danforth's short-tail* (*Sd*) mouse is a semidominant mutation that prevents completion of notochord development. In homozygous mutant mice, the notochord completely degenerates at embryonic day 9.5 (E9.5), whereas the neural tube and somites continue to form, permitting analysis of somite development in the absence of inductive signals from the notochord and floor plate. In the somites formed after notochord degeneration, *Myf5* expression initiates in a normal temporal sequence, but instead of the normal restriction to the dorsomedial somite, its expression extends into the ventral somite. *MyoD*, *myogenin*, and *myosin heavy chain* are normally expressed in the ventral myotome and there is normal development of hypaxial muscles. In contrast, subsequent to initial *Myf5* expression, muscle gene expression was not detected in the dorsal myotome and a high level of apoptosis was observed with significantly decreased formation of epaxial muscles. The apoptosis of epaxial muscle in somites that formed after notochord degeneration is consistent with a role for the notochord in the survival and differentiation of the dorsal myotome.** © 1998 Academic Press

***Key Words: Danforth's short-tail;* transgenic mice; myogenic bHLH; *MyoD; Myf5;* myogenesis; somite; myotome; notochord; *Sonic hedgehog;* apoptosis.**

INTRODUCTION

During vertebrate development, skeletal muscle is derived from the somites, epithelial spheres of the paraxial mesoderm formed in a rostrocaudal progression. In response to signals from the neural tube, notochord, and other surrounding tissues, the newly formed somites differentiate into a dorsal dermomyotome and a ventral sclerotome. The sclerotome forms the cartilage of the vertebral column and ribs and the dermomyotome gives rise to dermis and myotome (Christ and Ordahl, 1995; Balling *et al.*, 1996; Brand-Saberi *et al.*, 1996). The myotome contains two different trunk muscle lineages: cells of the dorsomedial myotome form the epaxial muscle (paraspinous muscles) and cells from the ventral myotome form the hypaxial muscles (abdominal wall and intercostal muscles). Cells from the ventral dermomyotome migrate to the developing limb buds to form the limb muscles (Rong *et al.*, 1992; Christ and Ordahl, 1995; Brand-Saberi *et al.*, 1996).

Signals from the dorsal neural tube, the dorsal ectoderm, and the lateral plate mesoderm regulate the development of the dermomyotome, mediated in part by Wnt and BMP proteins (Rong *et al.*, 1992; Munsterberg and Lassar, 1995; Munsterberg *et al.*, 1995; Stern *et al.*, 1995; Cossu *et al.*, 1996; Pourquie *et al.*, 1996; Xue and Xue, 1996; Dietrich *et al.*, 1997; Hirsinger *et al.*, 1997; Marcelle *et al.*, 1997). Signals from the notochord and floor plate stimulate the development of the sclerotome (Pourquie *et al.*, 1993; Koseki *et al.*, 1993; Dietrich *et al.*, 1993; Goulding *et al.*, 1994), mediated at least in part by the secreted Sonic hedgehog (Shh) protein (Fan and Tessier-Lavigne, 1994; Balling *et al.*, 1996; Brand-Saberi *et al.*, 1996). While recent experiments indicate that the Shh protein from notochord and floor plate induces both myogenesis and sclerogenesis (Munsterberg *et al.*, 1995; Hirsinger *et al.*, 1997; Marcelle *et al.*, 1997; Borycki *et al.*, 1998), the entire notochord has been shown to both positively (Munsterberg and Lassar,

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1995; Stern *et al.,* 1995; Pownall *et al.,* 1996; Xue and Xue, 1996; Dietrich *et al.,* 1997) and negatively (Pourquie, 1993; Bober *et al.,* 1994; Goulding *et al.,* 1994; Xue and Xue, 1996; Dietrich *et al.,* 1997) modulate myotome formation. Both notochord ablation experiments in avian embryos and targeted disruption of *Sonic hedgehog* (*Shh*) in mice have indicated a role for *Shh* and the notochord in the development of the epaxial myotome (Chiang *et al.,* 1996; Teillet *et al.,* 1998).

The members of the *MyoD* family of myogenic regulatory proteins (*MyoD, Myf5, myogenin, MRF4*) belong to the basic helix–loop–helix (bHLH) class of transcription factors and are expressed in skeletal muscle cell lineages (Davis *et al.,* 1987; Megeney and Rudnicki, 1995). *In situ* experiments in mouse embryos indicate that *Myf5* is the first myogenic bHLH gene to be expressed in the dorsomedial lip of the dermomyotome at embryonic day 8.0 (E8.0) (Lyons and Buckingham, 1992). The expression of *myogenin* and *MRF4* follow *Myf5* in the myotome, and subsequently *MyoD* expression begins at E10.0 in ventrolateral region of the myotomes at the trunk level (Sassoon *et al.,* 1989; Lyons and Buckingham, 1992; Smith *et al.,* 1994). Homologous recombination experiments demonstrated that mice with null mutations of *MyoD* or *Myf5* have a relatively normal muscle phenotype (Braun *et al.,* 1992; Rudnicki *et al.,* 1992). In contrast, mice that have null mutations of both *MyoD* and *Myf5* genes do not form any skeletal muscle, indicating that *MyoD* and *Myf5* can compensate for each other (Rudnicki *et al.,* 1993). Since *Myf5* is initially expressed in the dorsomedial myotome and *MyoD* in the ventrolateral myotome, some aspects of the compensation may be nonautonomous at the cellular level (Smith *et al.,* 1994; Braun and Arnold *et al.,* 1996; Cossu *et al.,* 1996).

Danforth's short-tail (*Sd*) mutation is a semidominant mutation that affects notochord development. The gene responsible for *Sd* mutation maps to proximal part of mouse chromosome 2 and has not yet been identified (Dunn *et al.,* 1940; Gluecksohn-Schoenheimer, 1945; Gruneberg, 1958). The initial notochord development is normal in *Sd* homozygotes until E9.5, when notochord formation ceases and the existing notochord rapidly degenerates. The neural tube continues to develop and new somites are formed in the caudal regions in the absence of a notochord (Gluecksohn-Schoenheimer, 1945; Gruneberg, 1958; Paavola *et al.,* 1980). As a consequence of the notochord defect, induction of floor plate in neural tube (Bovolenta and Dodd, 1991) and sclerotome (Dietrich *et al.,* 1993; Koseki, 1993) does not occur. In *Sd* heterozygotes, the degeneration of the notochord begins at a later stage and only tail development is affected (Paavola *et al.,* 1980; Gruneberg, 1958).

To examine dorsoventral specification of the somites that formed after notochord degeneration in *Sd* embryos, we analyzed the expression of muscle-specific genes in homozygous *Sd* mutant mouse embryos. In addition, the expression of a transgene, the *lacZ* gene driven by a 6-kb 5′ flanking sequence of the mouse *MyoD* gene (MD6.0-lacZ) was also used as a marker of skeletal muscle cell differentiation (Asakura *et al.,* 1995). Our studies show that the initiation of *Myf5* gene expression does not require the presence of the notochord, and that in the absence of a notochord, the expression of *Myf5* extends more ventrally than normal. While myotome formation is initiated in the absence of the notochord, the dorsal myotome, which forms the epaxial muscles, exhibits apoptosis and largely degenerates, whereas the ventral myotome, which forms the hypaxial muscles, develops relatively normally. The apoptosis of epaxial muscle in somites that formed after notochord degeneration is consistent with a role for the notochord in the survival and differentiation of the dorsal myotome, although a more direct role of the *Sd* gene product in somitogenesis cannot be ruled-out.

MATERIALS AND METHODS

Animals

Heterozygous RSV mice carrying *Danforth's short-tail* (*Sd*) mutation were obtained from the Jackson Laboratory. Heterozygotes were crossed and pregnant females were sacrificed on E9.0–E14.5. Before E10.0, it is difficult to distinguish between wild-type and mutant (*Sd*) embryos on the basis of their features. However, from E10.0, although it is still difficult to distinguish between wild type and heterozygotes, the tails of homozygous mutants (*Sd*) are slightly shrunken in the diameter and length, compared with heterozygous or wild-type litters. At E11.0, while heterozygotes exhibited slight constriction of the tails, homozygotes start to exhibit lumbosacral malformations (Gluecksohn-Schoenheimer, 1945; Gruneberg, 1958). After E11.0, the expected ratio of 1:2:1 was observed for wild-type:heterozygotes and homozygotes, respectively, according to their appearances. Furthermore, the expected ratio of 3:1 was observed for normal embryos (wild-type and heterozygotes):homozygotes, respectively, according to the *Sonic hedgehog* (*Shh*) expression (Roelink *et al.,* 1994) as a notochord/floor plate marker after E10.0.

Transgenic Mice

Transgenic mice carrying MD6.0-lacZ containing a 6-kb fragment from −5870 to +95 of the *MyoD* genomic fragment and bacterial *lacZ* gene were used for detecting muscle development (Asakura *et al.,* 1995). The 292 line, within four independent transgenic lines, most strongly expresses the *lacZ* gene in skeletal muscle cell lineage from E10.5. Heterozygotes or homozygotes from 292 line were crossed with *Sd* heterozygotes; subsequently, *Sd* heterozygotes carrying the transgene were used to obtain *Sd* homozygotes carrying the transgene to characterize muscle differentiation in the mutant background.

RNA Probes

Digoxigenin-11-UTP-labeled antisense cRNA probes used in this study were prepared as follow. The probe for rat *Sonic hedgehog* (*Shh*) corresponds to the entire coding cDNA sequence, 2.5-kb fragment (Roelink *et al.,* 1994). The probe for mouse *Pax1* corresponds to the entire coding sequence, 1.0-kb fragment (Koseki *et*

al., 1993). The probe for mouse *Pax3* corresponds to the 516-bp *Hin*dIII–*Pst*I fragment (Bober *et al.,* 1994). The probe for mouse *Myf5* corresponds to the entire coding sequence, 760-bp fragment (Yoon *et al.,* 1997). The probe for mouse *MyoD* corresponds to the 1035-bp (751–1785) fragment (Sassoon *et al.,* 1989). The probe for mouse *myogenin* corresponds to the 696-bp (791 to 1486) fragment (Sassoon *et al.,* 1989). The probe for mouse *MRF4* corresponds to the 500-bp *Sac*I–*Xba*I genomic DNA fragment including the third exon (Miner and Wold, 1990). The probe for mouse *myosin heavy chain* (*MHC*) corresponds to the 3′ part of the cDNA, 700-bp fragment (Davis *et al.,* 1987).

Whole Mount in Situ Hybridization

The whole mount *in situ* hybridization was performed as described previously (Bober *et al.,* 1994). Hybridization was performed with digoxigenin-11-UTP labeled cRNA probes at 65°C overnight and detected with preabsorbed alkaline phosphatase-coupled anti-digoxigenin antibodies (Boehringer Mannheim) followed by reaction with NBT and BICP or BM purple (Boehringer Mannheim). For vibratome sections, the samples after staining were rinsed in PBT briefly and then postfixed overnight in 4% paraformaldehyde at 4°C. Following fixation, embryos were rinsed in PBT and then embedded in 20% gelatin in PBT. Fifty- to 100-μm sections were made using Vibratome 1000 (TPI).

Whole Mount TUNEL Staining

Apoptotic cell death was detected in whole embryos by a modification of the TUNEL procedure (Conlon *et al.,* 1995). Embryos were incubated with 20 μM digoxigenin-dUTP, 20 μM dTTP, 0.3 U/μL terminal transferase in TdT buffer (30 mM Tris–HCl, 140 mM cacodylate, pH 7.2, 1 mM $CoCl_2$) at 37°C for 2 h. The stainings were detected with alkaline phosphatase-coupled anti-digoxigenin antibodies (Boehringer Mannheim) followed by reaction with NBT and BICP. Fifty- to 100-μm sections were made using Vibratome 1000 (TPI).

β-Galactosidase Staining

Whole mount β-galactosidase staining was performed as described previously (Asakura *et al.,* 1995). Fifty- to 100-μm sections were made using TPI Vibratome 1000.

RESULTS

Perturbation of Sonic hedgehog (Shh) Expression in the Notochord and Floor Plate in Sd Embryos

We cannot verify the genotype of the *Sd* embryos conclusively because the *Sd* gene has not been identified. Before E10.0, it is difficult to distinguish between wild-type and mutant embryos on the basis of their phenotype. Beginning at E10.0, however, the tails of homozygous mutants are smaller in diameter and length compared with normal (heterozygous or wild-type) litter mates (Gluecksohn-Schoenheimer, 1945; Gruneberg, 1958). Based on this genotyping criteria, the expected ratio of 3:1 was observed for normal (wild-type/heterozygous):homozygous embryos from E10.0 to E11.0. To verify that the short tailed embryos had abnormal notochord development, we analyzed expression of *Shh* by whole mount *in situ* hybridization as a molecular marker of notochord formation.

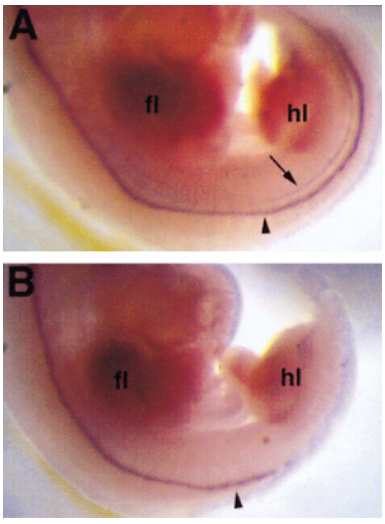

FIG. 1. The expression of *Sonic hedgehog* (*Shh*) gene in wild-type and *Sd* homozygous embryos at E10.5. At E10.5, *in situ* hybridization to *Shh* was observed in the ventral axial structures, notochord (arrow) and floor plate (arrowhead), extending rostrally to caudally in wild-type embryos (A). In *Sd* embryos, *Shh* expression was not detected in the axial structures in the caudal (lumbosacral) region or in the region where the notochord had degenerated, but was observed in the rostral (cervical and thoracic) floor plate (arrowhead) that had been induced prior to notochord degeneration (B). hl; hindlimb bud.

Shh is expressed initially in the notochord and induces the formation of the neural tube floor plate, which also expresses *Shh* (Roelink *et al.,* 1994). In normal embryos, *Shh* is expressed along the entire notochord from E7.5 and the floor plate in the ventral neural tube from E8.5 (Weinstein *et al.,* 1994). Until E9.5, *Sd* embryos have a normal *Shh* expression pattern; however, *Shh* expression disappears after E9.5, coincident with the degeneration of the notochord (Fig. 1; Gluecksohn-Schoenheimer, 1945; Gruneberg, 1958). Interestingly, in *Sd* embryos, *Shh* expression persists in the cervical and thoracic regions where floor plate induction occurred prior to notochord degeneration (Figs. 1B, 2B, 2D, and 2J) Therefore, the floor plate length in *Sd* embryos serves as a marker for the region induced by the notochord prior to its degeneration (Bovolenta and Dodd, 1991) and ranges between the 17th and 23rd somites (data not shown). The absence of floor plate induction in the caudal region indicates that the somites in this region have not received signals from either the notochord or floor plate, an important point since floor plate is able to substitute for notochord in some experiments of somite specification.

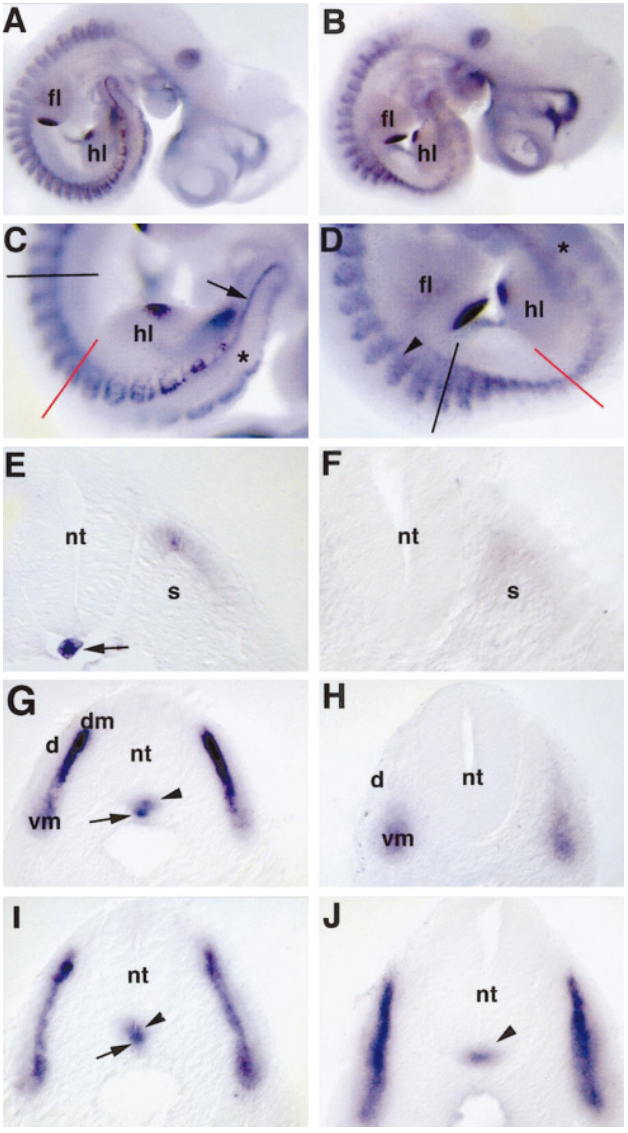

FIG. 2. The expression of *Myf5* and *Shh* genes in wild-type and *Sd* embryos at E10.0. At E10.0, wild-type (A, C, E, G, I) and *Sd* (B, D, F, H, J) embryos were stained for *Shh* and *Myf5* using whole mount *in situ* hybridization. Simultaneous use of both probes was performed to confirm *Sd* homozygotes. *Shh* expression was detected in the brain, the posterior mesenchyme of the limb buds, and the hindgut in wild-type (A, C) and *Sd* (B, D) embryos. Wild-type embryos (C, E, G, I) expressed *Shh* in the notochord (arrow) and floor plate (arrowhead). In *Sd* embryos, *Shh* was not detected in the notochordal region (D, F, H, J) or caudal floor plate region (D, F, H) but was detected in the cervical and thoracic floor plate (arrowhead in D, J). In wild-type embryos (A, C, E, G, I), *Myf5* expression was observed in the entire myotome from the cervical to the lumber region and in the dorsomedial lip of the newly formed somites in the sacral region (E and asterisk in C). In *Sd* embryos, *Myf5* was normally expressed in entire myotome in the cervical and thoracic regions (B, D, J) but the expression was reduced in the dorsomedial myotome in the lumbar and sacral regions (B, H). In the newly formed somites of the sacral region, the expression expanded more ventrally than normal (F and asterisk in D). C and D show high power views from A and B, respectively. Transverse sections of the embryos at tail bud level (E, F), lumber level (G, H), and thoracic level (I, J), indicated by asterisks, red bars, and black bars, respectively, in C and D. fl, forelimb bud; hl; hindlimb bud; nt; neural tube; s, segmental somite; d, dermomyotome; dm, dorsal myotome; vm, ventral myotome.

Perturbation of Dorsoventral Somite Specification in Sd Embryos at E10

To examine dorsoventral specification of the somites that formed after notochord degeneration in *Sd* embryos, we analyzed the expression patterns of the *Myf5* and *Pax1* genes. In normal embryos, *Myf5* first appears in the dorsomedial lip of the newly formed somite and subsequently is expressed throughout the myotome (Figs. 2A, 2C, 2E, 2G, and 2I; Lyons and Buckingham, 1992; Yoon *et al.*, 1997). At E10.0 *Myf5* expression is present along the entire body axis up to the most newly formed somite (29 segments) (Figs. 2A, 2C, and 2E). In the most caudal regions, *Myf5* transcripts are detected in the dorsomedial lip of the newly formed myotome (the epaxial domain) (Figs. 2C and 2E), whereas in the cervical and thoracic regions *Myf5* is expressed throughout the myotome (epaxial and hypaxial domains) (Figs. 2C and 2I). In *Sd* embryos, *Myf5* expression was normal at E9.5, prior to notochord degeneration (data not shown). At E10, however, in the most caudal somites *Myf5* expression expanded ventrally and was slightly reduced in intensity compared to normal embryos (Fig. 2: compare B, D, and F to A, C, and E), suggesting that the signal from the notochord and floor plate is not necessary for the initial induction of *Myf5* expression but is necessary to maintain the dorsomedial localization and to achieve normal levels of expression. *Myf5* expression was also significantly reduced in the dorsal region of the lumbosacral myotomes (18th to 27th) of *Sd* embryos compared to wild-type embryos. In wild-type embryos, *Myf5* expression was higher in the dorsal than the ventral myotome, whereas in *Sd* embryos the *Myf5* was expressed ventrally, but not dorsally (Fig. 2: compare C, G and D, H). In the rostral myotomes (1st to 17th), *Myf5* expression appeared unaffected in the *Sd* embryos (Figs. 2I and 2J). Therefore, *Myf5* expression was diminished in the dorsal myotome of somites that formed in the absence of signals from the notochord and floor plate, whereas the ventral myotome showed relatively normal development.

Pax1, a paired box gene, is expressed in the developing sclerotome with transcripts first appearing in the ventral part of the newly formed somite (Figs. 3A and 3C; Balling *et al.*, 1996). At E9.5, *Pax1* expression was normal in *Sd* embryos (data not shown); however, at E10.0 in the most caudal somites, *Pax1* expression was significantly diminished compared to wild-type embryos (Figs. 3B and 3D; Dietrich *et al.*, 1993; Koseki *et al.*, 1993), consistent with

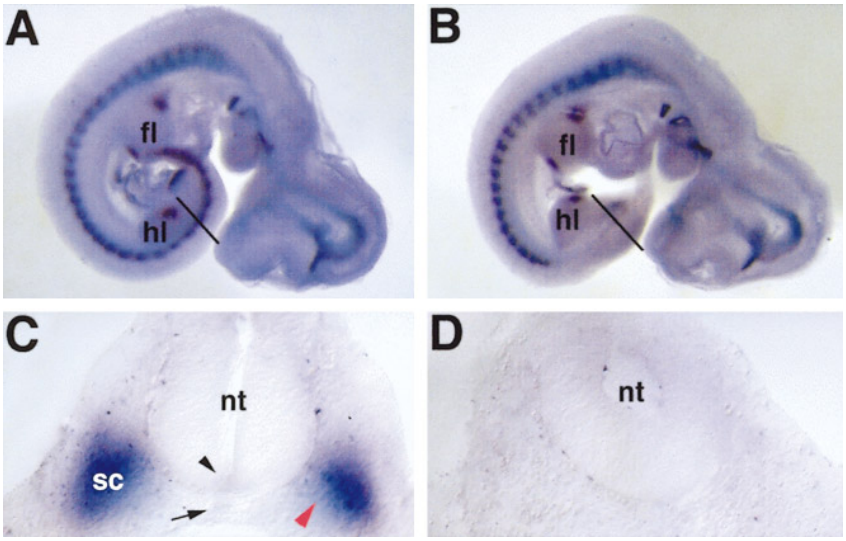

FIG. 3. The expression of *Pax1* and *Shh* genes in wild-type and *Sd* embryos at E10.0. At E10.0, wild-type (A, C) and *Sd* (B, D) embryos were stained for *Shh* and *Pax1* by whole mount *in situ* hybridization. To confirm *Sd* homozygotes, both probes were used for the same embryos in each whole mount *in situ* hybridization. Wild-type embryos (A, C) expressed *Shh* in the notochord (arrow) and floor plate (arrowhead). In *Sd* embryos, *Shh* expression was absent in the entire notochordal region and caudal floor plate (B, D). In wild-type embryos (A, C), *Pax1* was observed in entire sclerotome (red arrowhead) from the cervical to the tailbud region. In *Sd* embryos, *Pax1* was normally expressed in entire sclerotome of the rostral region (B) but was not expressed in the sclerotomal region in caudal somites (B, D). Transverse sections of embryos at the sacral level (C, D) are indicated by bars in A and B. fl, forelimb bud; hl; hindlimb bud; nt; neural tube; sc, sclerotome.

ablation studies in avian embryos that indicate a role of the ventral axial structures (notochord and floor plate) in sclerotome development (Borycki *et al.*, 1997; Dietrich, *et al.*, 1997; Teillet *et al.*, 1998). Together, these data are consistent with the model that ventral axial structures play a role in the dorsoventral specification of the somite, restricting the domain of *Myf5* expression to the dorsal somite, maintaining *Myf5* expression in the dorsal myotome, and inducing sclerotome and *Pax1* expression in the ventral somite.

Perturbation of Dorsal Myotome Differentiation in Sd Embryos at E10

To analyze muscle cell differentiation in the *Sd* myotomes, we examined the expression of the *myosin heavy chain* (*MHC*) gene. *MHC* is an early marker of the postmitotic muscle cell in the myotome and its expression continues in differentiated muscle fibers (Lyons, 1990; Lyons and Buckingham, 1992). At E10.0, *MHC* expression in wild-type embryos was detected in the middle of the most caudal differentiating myotomes (29th of 35 somites) (Fig. 4A, asterisk; Fig. 4C, arrowhead) and expanded to the dorsal and ventral parts of myotome more rostrally. Similar to wild type, in *Sd* embryos at E10.0, *MHC* was detected in the middle of the most caudal differentiating myotome (28th of 34 somites) (Fig. 4B, asterisk; Fig. 4D, arrowhead) and was normally expressed in the cervical and thoracic myotomes (1st to 20th) (compare arrow in Figs. 4A and 4B). In contrast to wild-type embryos, however, *MHC* expression was significantly reduced in the dorsal region of the caudal myotomes that had developed after notochord degeneration (23rd to 27th myotomes) (compare bar in Figs. 4A and 4B; and Fig. 4E and 4F). Therefore, caudal myotomes with diminished dorsal expression of *Myf5* (see Figs. 2G and 2H) also had diminished *MHC* expression, indicating an absence of muscle cell differentiation. Therefore, the differentiation of the dorsal myotome did not progress in the absence of the ventral axial structures of the notochord and floor plate.

It is interesting that the initial *MHC* expression occurred normally, since these cells are thought to originate from the dorsomedial lip of the dermomyotome and subsequently migrate to the midmyotome position where they initiate *MHC* expression (Lyons and Buckingham, 1992). This would indicate that the initial myoblast formation occurs in the dorsal dermomyotome in the absence of notochord signaling, but that subsequent differentiation in the dorsal myotome does not occur. However, we cannot rule out the possibility that the initial *MHC*-expressing cells migrated from the ventral edge of the dermomyotome in *Sd* embryos.

Expression of MyoD and myogenin in Sd Embryos at E10.0

In wild-type embryos at E10.0, *MyoD* is expressed in the ventrolateral myotome of the thoracic and lumbosacral myotomes (Figs. 5C, 5E, and 5G; Smith *et al.*, 1994) and *myogenin* is expressed in both the dorsomedial and ventro-

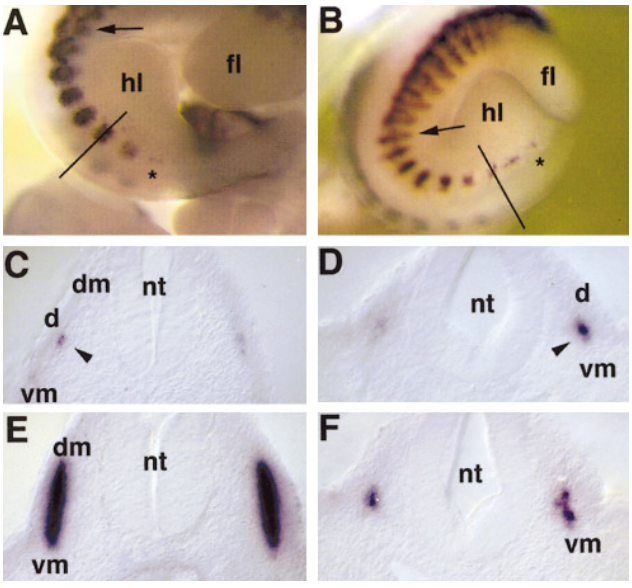

FIG. 4. Expression of the *myosin heavy chain* (*MHC*) gene in wild-type and *Sd* embryos at E10.0. At E10.0, wild-type (A, C, E) and *Sd* (B, D, F) embryos were stained for *MHC* by whole mount *in situ* hybridization. *MHC* expression in wild-type embryos (A, E) was observed in the entire myotome in the rostral to caudal regions (arrow and bar) but was restricted at the center of newly formed myotomes in tail bud region (arrowhead in C and asterisk in A). In *Sd* embryos, *MHC* expression was detected in the entire myotome in the rostral regions (arrow in B); however, in the caudal regions, *MHC* expression was significantly reduced in the dorsal part of myotome (F and bar in B). Like wild-type embryos, *MHC* expression in *Sd* embryos was restricted at the center of newly formed myotome in the tail bud region (arrowhead in D and asterisk in B). Transverse sections of the embryos at tailbud level (C, D) and sacral level (E, F) indicated by asterisks and bars in A and B, respectively. fl, forelimb bud; hl; hindlimb bud. nt; neural tube; d, dermomyotome; dm, dorsal myotome; vm, ventral myotome.

lateral regions of the myotome (Fig. 5A; Smith *et al.*, 1994). In *Sd* embryos the expression of *MyoD* was not significantly altered (Figs. 5D, 5F, and 5H), whereas *myogenin* expression was reduced in the dorsomedial myotome and normal in the ventrolateral myotome (Fig. 5B). Similarly, *MRF4* expression was reduced in the dorsomedial myotome (data not shown). Since both *myogenin* and *MRF4* are thought to be activated by *Myf5* and *MyoD* (Megeney and Rudnicki, 1995), the decreased expression in the dorsomedial myotome may be secondary to the reduced levels of *Myf5;* and the normal expression in the ventrolateral myotome may reflect the normal levels of *MyoD* expression. Therefore, in somites that develop in the absence of the notochord, the Sd mutation has profound consequences for the *Myf5*-expressing dorsal myotome that will give rise to epaxial muscle but does not significantly affect the *MyoD*-expressing ventral myotome that will give rise to hypaxial muscle, a phenotype consistent with the proposed role of the ventral axial structures in promoting epaxial differentiation.

Decline of the Expression of Muscle-Specific Genes in Maturing Myotomes of Sd Embryos at E11.5–E12.5

In the caudal myotomes of *Sd* embryos at E11.5, *MyoD, myogenin, Myf5,* and *MHC* expression was restricted to the ventral most region (Figs. 6B, 6D, 6F, and 6H and data not shown), while in wild-type embryos the entire myotome expressed these genes (Fig. 6A, C, E, G and data not shown). Similar results were obtained from whole mount immunohistochemistry using anti-MHC antibodies (data not shown). In addition to the absence of the expression of muscle genes in the dorsal myotome, the expression in the ventral myotome was shifted slightly medially to a position that would normally be occupied by sclerotome in wild-type embryos (see Fig. 6H). These results are consistent with the model that the dorsal (epaxial) myotome is dependent on the presence of the ventral axial structures for normal differentiation, whereas the most ventral (hypaxial) myotome can maintain differentiation in the absence of the ventral axial structures. In this regard, it is interesting that while somitogenesis was disrupted in the *Sd* embryos, differentiation of most ventral myotome, the forelimbs, hindlimbs, and face appeared to progress normally as determined by expression of *MyoD, myogenin,* and *MHC* (Fig. 6 and data not shown).

Decreased MyoD-lacZ Expression in the Caudal Epaxial Muscles of Sd Embryos

We have previously demonstrated that transgenic mice driving a *lacZ* reporter gene from the 6-kb upstream region of the mouse *MyoD* gene express the transgene in differentiating skeletal muscle (Asakura *et al.*, 1995). Therefore, the muscle differentiation in these transgenic mice can be followed during embryogenesis by using whole mount lacZ staining. In wild-type embryos at E11.5, lacZ expression was detected in dorsal and ventral region of both the rostral and caudal myotomes (Figs. 7A and 7C). In *Sd* embryos, lacZ expression was similar to wild type in the rostral myotomes, but substantially reduced in the dorsal region of the caudal myotomes (Figs. 7B and 7D), consistent with the endogenous *MyoD* expression pattern (Figs. 6A and 6B). At E14.5 in wild-type embryos, lacZ expression was detected in most of the muscle masses, including intercostal, back, and hindlimb muscles (Fig. 7E). In *Sd* embryos, lacZ expression was substantially reduced in the caudal epaxial muscle masses (back muscles), but was expressed in the hypaxial and hindlimb muscles (Fig. 7F). The lacZ-positive hypaxial muscles are thought to originate from the ventral myotomes. Therefore, at later developmental ages, epaxial muscles derived from the dorsal myotome were severely affected in the *Sd* embryos, whereas hypaxial and limb muscles derived from the ventral myotome were relatively unaffected.

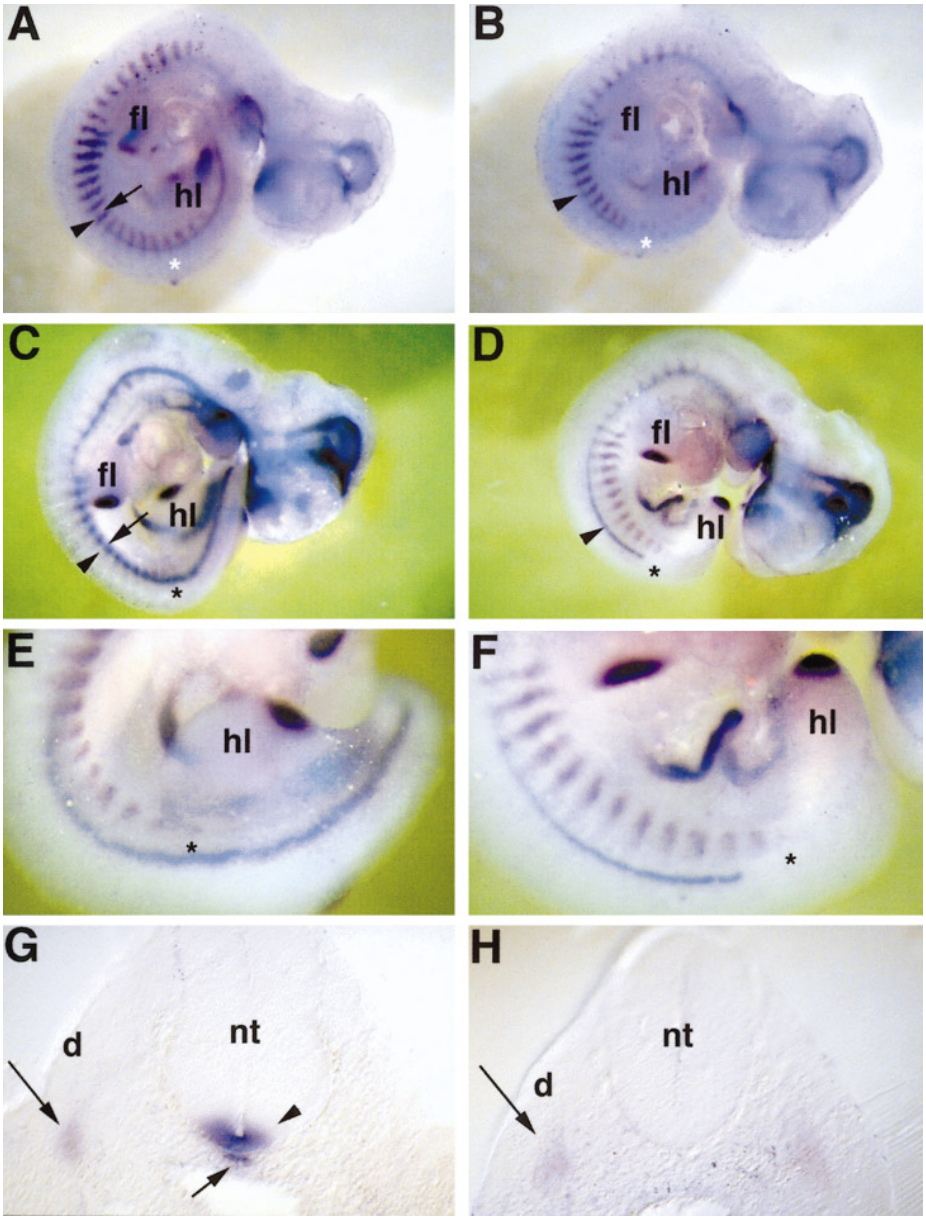

FIG. 5. Expression of the myogenic bHLH genes *myogenin* and *MyoD* in wild-type and *Sd* embryos. At E10.5, normal (A, C, E, G) and *Sd* (B, D, F, H) embryos were stained for *myogenin* (A, B) and *MyoD* (C, D, E, F, G, H) by whole mount *in situ* hybridization. To confirm *Sd* homozygotes, *Shh* probes were used simultaneously. Wild-type embryos (A, C, E, G) expressed *Shh* in the notochord (short arrow) and floor plate (arrowhead). In *Sd* embryos *Shh* was not expressed in the entire notochordal region and caudal floor plate region (B, D, F, H). *Myogenin* was observed in the entire myotome in the rostral region in both wild-type (A) and *Sd* (B) embryos. In the caudal myotomes, *myogenin* was expressed in the entire myotome in wild-type embryos, but was expressed only in the ventral myotome *Sd* embryos (asterisk in A and B). *MyoD* expression was observed in the dorsal myotomes in the cervical region in both wild-type (C) and *Sd* (D) embryos. In addition, *MyoD* expression was observed in the ventral myotome from the forelimb bud to the hindlimb bud region in both wild-type and *Sd* embryos (asterisk in C, D, E, and F and long arrow in G and H). Transverse sections of the embryos at the hindlimb bud level (G, H), indicated by asterisks in C, D, E, and F. fl, forelimb bud; hl; hindlimb bud. nt; neural tube; d, dermomyotome.

Expression of Pax3 in Wild-Type and Sd Embryos

Myotomal cells differentiate and migrate into the myotome from the dorsal and ventral edge of the dermomyotome (Christ and Ordahl, 1995; Denetclaw *et al.*, 1997). To determine whether the reduction of the dorsal myotome in *Sd* embryos resulted from a defect of the dermomyotomes, we examined *Pax3* expression in *Sd* embryos. *Pax3* is expressed in the caudal segmental plate and becomes re-

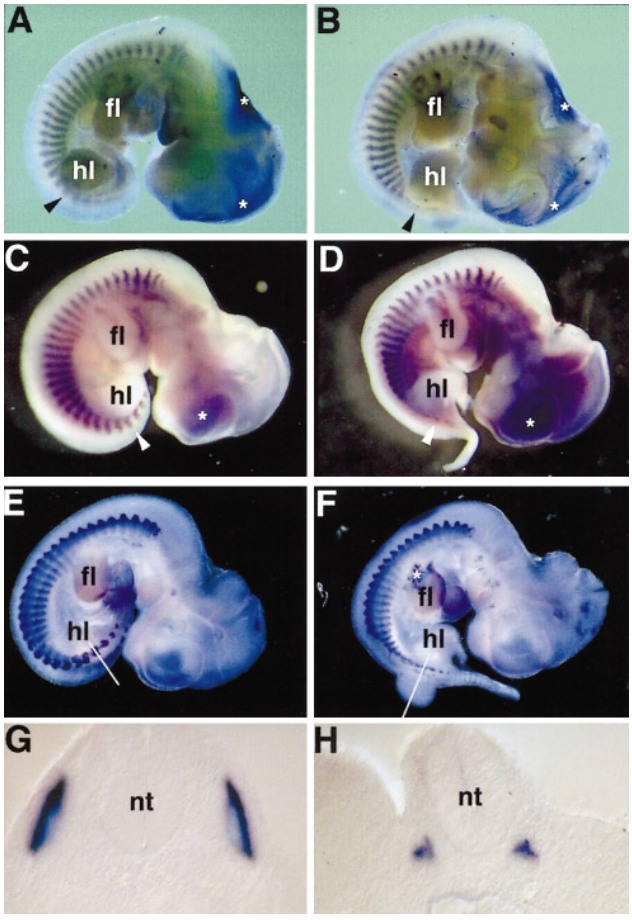

FIG. 6. Expression of *MyoD, myogenin,* and *MHC* in wild-type and *Sd* embryos at E11.5. At E11.5, wild-type (A, C, E, G) and *Sd* (B, D, F, H) embryos were stained for *MyoD* (A, B), *myogenin* (C, D), and *MHC* (E, F, G, H) by whole mount *in situ* hybridization. In wild-type embryos, *myogenin, MyoD,* and *MHC* were observed in the myotomes, the skeletal muscle of the forelimb and hindlimb buds, and the facial muscles (A, C, E, G). In *Sd* embryos, all three genes were expressed in the entire myotome in the rostral region, but were significantly decreased in the dorsal myotome in the caudal region (arrowhead in B and D, bar in F) compared to wild-type embryos (arrowhead in A and C, bar in E). The expression of these three genes in the forelimb bud, hindlimb bud, and facial muscles was not significantly affected in *Sd* homozygotes. Transverse sections of the embryos at hindlimb bud level (G, H), indicated by bars in E, F. fl, forelimb bud; hl, hindlimb bud; nt, neural tube; asterisks, nonspecific staining.

stricted to the dermomyotome with somite maturation (Bober *et al.,* 1994; Goulding *et al.,* 1994; Williams and Ordahl, 1994). At E10.0, wild-type embryos expressed *Pax3* in the dermomyotomes, segmental somites, and dorsal neural tube (Figs. 8A, 8C, and 8E). In *Sd* embryos, *Pax3* was normally expressed in the somites and dermomyotomes, but expression in neural tube extended further ventrally than normal (Figs. 8B, 8D, and 8F; Koseki *et al.,* 1993). At E11.5, *Pax3* expression was diminished in the dorsal region of the dermomyotome of Sd embryos (Dietrich *et al.,* 1993 and data not shown). Based on *Pax3* expression, therefore, the initial development of the dermomyotome is not significantly affected by the absence of a notochord and floor plate, suggesting that the loss of *Myf5* expression in the dorsal myotome is not due to the failure of the dermomyotome to develop. Signals from the notochord may be required for maintenance of the dorsal dermomyotome; however, since *Pax3* expression was lost in the dorsal dermomyotome at E11.5.

Apoptotic Cell Death in the Dorsal Myotome, Dorsal Dermomyotomes, and Dorsal Ectoderm of Sd Embryos

We have shown the initial development of the dermomyotome and myotome occurred in the newly formed somites of *Sd* embryos, based on initial *Myf5* and *Pax3* expression. During the period that the dorsal myotome should be maturing, however, the expression of *Myf5* and *Pax3* is lost, and the dorsal myotome failed to express markers of differentiation, such as *myogenin, MRF4, MyoD,* and *MHC.* We used TUNEL staining to determine whether the reduction of muscle gene expression in the dorsal myotome was associated with apoptosis of cells in the dorsal myotome and dermomyotome. In wild-type embryos, TUNEL staining detected a small amount of apoptosis in the caudal somites at E10.5 (Figs. 9A and 9C). In contrast, *Sd* embryos (Figs. 9B and 9D) showed extensive apoptotic cell death that was largely restricted to the dorsal ectoderm, dorsal dermomyotome and dorsal myotome, sparing the neural tube, ventral dermomyotome, and ventral myotome where *MyoD*-expressing cells first differentiate from the dermomyotome (Figs. 5G and 5H).

DISCUSSION

Many of the recent studies of the role of the notochord in somitogenesis have used ablation or implantation techniques *in vivo,* or in coculture systems *in vitro.* Possibly because of the different experimental systems and species used, the role of the notochord on dorsoventral patterning of the paraxial mesoderm and on myotome development has been controversial, but a general model has emerged that suggests a role of the ventral axial structures in supporting epaxial myotome differentiation and survival (Currie and Ingham, 1998; Teillet *et al.,* 1998).

In this study, we analyzed skeletal muscle development in somites formed after notochord degeneration in *Danforth's short-tail* mice (*Sd*). In the *Sd* mutant the rostral notochord degenerates at E9.5 and the caudal notochord fails to form. Results from our study are consistent with the model that signals from the notochord and floor plate, the ventral axial structures, contribute to the restriction of initial *Myf5* expression to the dorsomedial lip of the der-

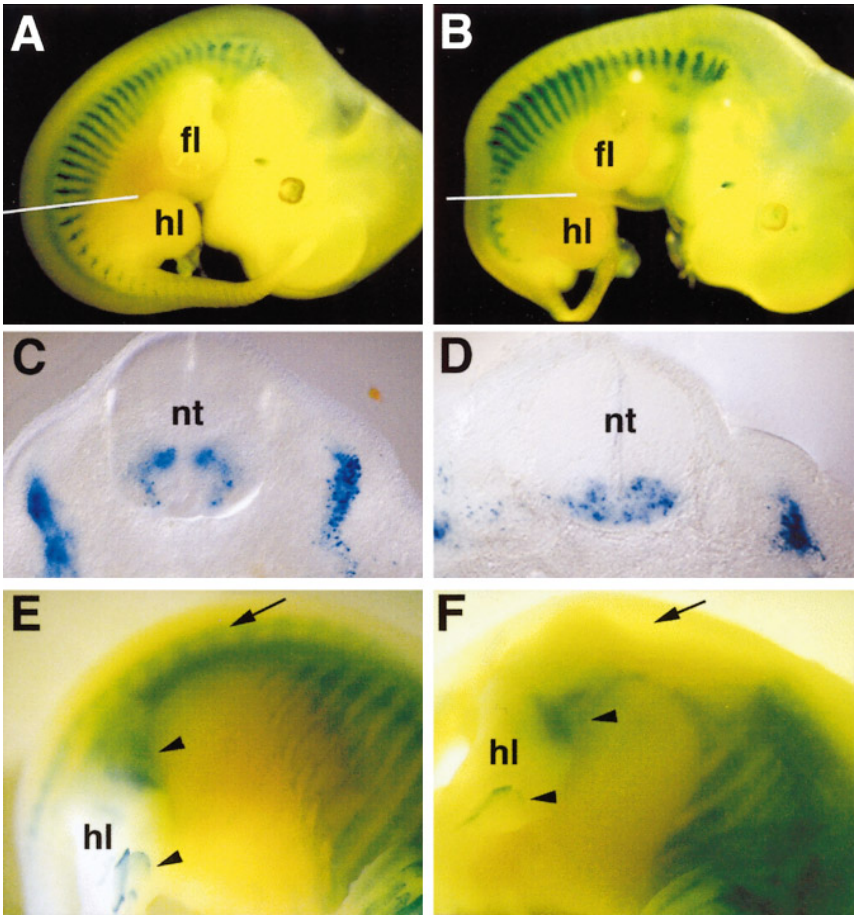

FIG. 7. Comparison of the expression patterns of the MD6.0-lacZ transgene in wild-type and *Sd* embryos. Wild-type (A, C, E) and *Sd* (B, D, F) mice with the MD6.0-lacZ transgene were analyzed by whole mount X-gal staining at E11.5 (A, B, C, D) and E14.5 (E, F). In the wild-type embryos at E11.5, lacZ expression was detected in the entire myotome both rostrally and caudally (A, C). In the *Sd* embryos at E11.5, expression was detected in the entire rostral myotome; however, in the caudal region expression was significantly reduced in the dorsal myotome (B, D). Transverse sections of the embryos at caudal regions (C, D; indicated by bars in A and B). Ectopic lacZ expression was detected in the ventral neural tube of both wild-type and *Sd* embryos. In wild-type embryos at E14.5 (E), lacZ expression was detected in the intercostal, back (arrow) and hindleg (arrowhead) muscles. In *Sd* embryos at E14.5 (F), lacZ expression was detected in the intercostal and hindleg (arrowhead) muscles; however, expression was significantly reduced in the back muscles (arrow). fl, forelimb bud; hl, hindlimb bud; nt, neural tube.

momyotome and to cell survival and differentiation in the dorsal myotome.

Danforth's short-tail (Sd) Mutant

The *Sd* mutation provides the opportunity to observe mouse somitogenesis after notochord degeneration. Since the mutation has not yet been identified, it is important to note that some aspects of the somite phenotype in these mice could be direct consequences of the mutation. Several lines of evidence, however, suggest that the *Sd* mutation primarily affects notochord development and that the abnormal development of the paraxial mesoderm and neural tube are secondary. First, the notochord has been shown to be the first structure visibly affected in mutant embryos (Gluecksohn-Schoenheimer, 1945; Gruneberg, 1958; Paavola *et al.*, 1980). Second, in *Sd* heterozygous embryos the notochord degenerates at a later stage and there is no skeletal muscle phenotype (A.A. and S.J.T., unpublished data). Third, the severity of the paraxial mesoderm and neural tube defects in *Sd* homozygous embryos correlates with the embryonic stage at which notochord degeneration begins (Gluecksohn-Schoenheimer, 1945; Gruneberg, 1958). Fourth, the mutation acts cell autonomously in the cells of the notochord and ventral hindgut as determined by chimeric mouse studies (Maatman *et al.*, 1997). While the cells of the ventral hindgut may be primarily affected by the *Sd* mutation, these cells have not been implicated in somitogenesis. Fifth, a similar correlation between

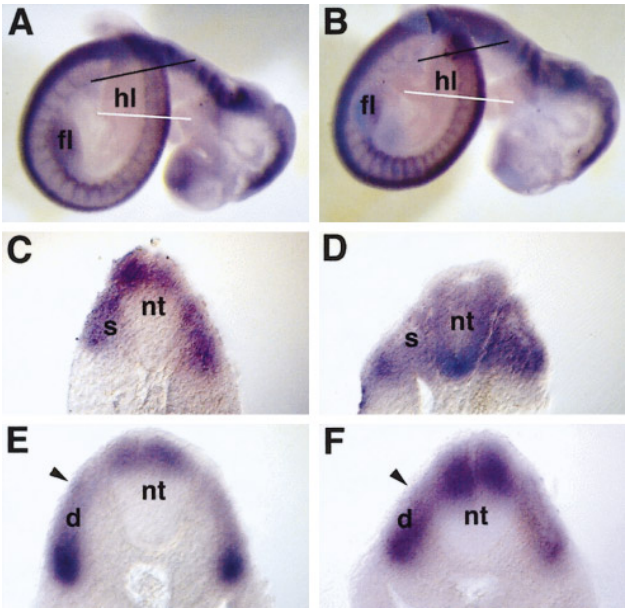

FIG. 8. Expression of *Pax3* in wild-type and *Sd* embryos at E10.0. Wild-type (A, C, E) and *Sd* (B, D, F) embryos were stained for *Pax3* by whole mount *in situ* hybridization. In wild-type embryos, *Pax3* expression was observed in the segmental somites at the tail bud region (C and black bar in A) and the hindlimb region (E and white bar in A), as well as in the dorsal neural tube and the dermomyotome. In *Sd* embryos, *Pax3* expression was detected in segmental somites similar to the wild-type embryo, but the expression in the neural tube in the tail bud region (D and black bar in B) and in the hindlimb bud region (F and white bar in B) extended significantly more ventrally. Arrowheads show weak *Pax3* expression in the dorsal dermomyotomes in normal (E) and *Sd* homozygous (F) embryos. fl, forelimb bud; hl, hindlimb bud; nt, neural tube; s, segmental somite; d, dermomyotome.

notochord changes and paraxial mesoderm development was observed *Brachyury curtailed* (T^c), a mutation that suppresses the expression of the *brachyury T* transcription factor in the notochord (Dietrich *et al.*, 1993). Sixth, recent studies ablating the notochord in avian systems (van Straaten and Hekking, 1991; Goulding *et al.*, 1994; Dietrich *et al.*, 1997) or knocking-out the *Shh* gene in mice (Chiang *et al.*, 1996) both reported a neural tube and somite phenotype similar to that of the *Sd* mouse (Bovolenta and Dodd, 1991; Dietrich *et al.*, 1993; Koseki *et al.*, 1993 and this paper). Seventh, at the time this manuscript was submitted, Teillet *et al.* (1998) showed that Shh from the avian notochord was necessary for cell survival in the epaxial myotome. Therefore, the phenotype of the rostral somites in the *Sd* mice is similar to that produced by notochord excision in avian embryos. Together, these observations support the interpretation that the abnormal dorsoventral specification of the somite and the lack of differentiation of the dorsal myotome in *Sd* embryos are secondary to the degeneration of the notochord.

Ventral Specification of the Somite, but Not Initial Myogenic Induction, Is Disrupted in Rostral Somites of Sd Embryos

Recent molecular analyses demonstrate that dorsomedial and ventrolateral specification of the somite is determined by the balance of signals from multiple structures: the notochord and floor plate (via Shh), the dorsal neural tube (via Wnt1, Wnt3, Wnt3a), the dorsal ectoderm (unidentified), the lateral plate (via BMP4), and the somite itself (via Noggin, Follistatin, Wnt11) (Munsterberg and Lassar, 1995; Munsterberg *et al.*, 1995; Stern *et al.*, 1995; Amthor *et al.*, 1996; Cossu *et al.*, 1996; Hirsinger *et al.*, 1997; Marcelle *et al.*, 1997; Borycki *et al.*, 1998). The signals from the dorsal neural tube and dorsal ectoderm induce *Pax3* gene expression and stimulate development of the dermomyotome, acting to dorsalize the somite (Maroto *et al.*, 1997). The signals from the ventral axial structures (notochord and floor plate) induce *Pax1* gene expression and stimulate development of the sclerotome, acting to ventralize the somite (Balling *et al.*, 1996). The dorsalizing factors and the ventralizing factors act in concert to induce *Myf5* gene expression and stimulate development of the myotome (Munsterberg and Lassar, 1995; Munsterberg et al. 1995; Dietrich *et al.*, 1997; Hirsinger *et al.*, 1997).

In the *Sd* embryo the notochord was not essential for the initiation of muscle gene expression in the somite since *Myf5*, the first myogenic bHLH gene expressed in the mouse somite, was expressed in newly formed somites after notochord degeneration, although the expression was reduced in intensity. In contrast to the restriction of *Myf5* expression to the dorsomedial lip of the dermomyotome, in the *Sd* embryos *Myf5* expression extended into the ventral somite. Conversely, *Pax1* expression was reduced in the ventral somites in *Sd* embryos. Therefore, while the ventral axial structures were not required for the initial expression of *Myf5*, ventral specification did not occur in somites that formed after notochord degeneration in the Sd embryos, consistent with a proposed role of these structures in ventral specification of the newly formed somite (Goulding *et al.*, 1994).

Decreased Survival and Differentiation in the Dorsal Myotome of Somites That Form after Notochord Degeneration in Sd Embryos

Shh protein has been implicated as the notochord-derived signal that cooperates with Wnt proteins from the dorsal neural tube to induce sclerogenesis and myogenesis in the somite (Munsterberg *et al.*, 1995; Hirsinger *et al.*, 1997; Marcelle *et al.*, 1997; Borycki *et al.*, 1998). Our data are consistent with the model that the Shh signal from the notochord is not necessary to initiate the expression of lineage genes in the myotome, but rather that the notochord supports the survival and differentiation of those cells directly or mediated by the dorsal dermomyotome and/or dorsal ectoderm. This result is consistent with the analysis of mice with a homozygous targeted disruption of the *Shh* gene (Chiang *et al.*, 1996) and with notochord

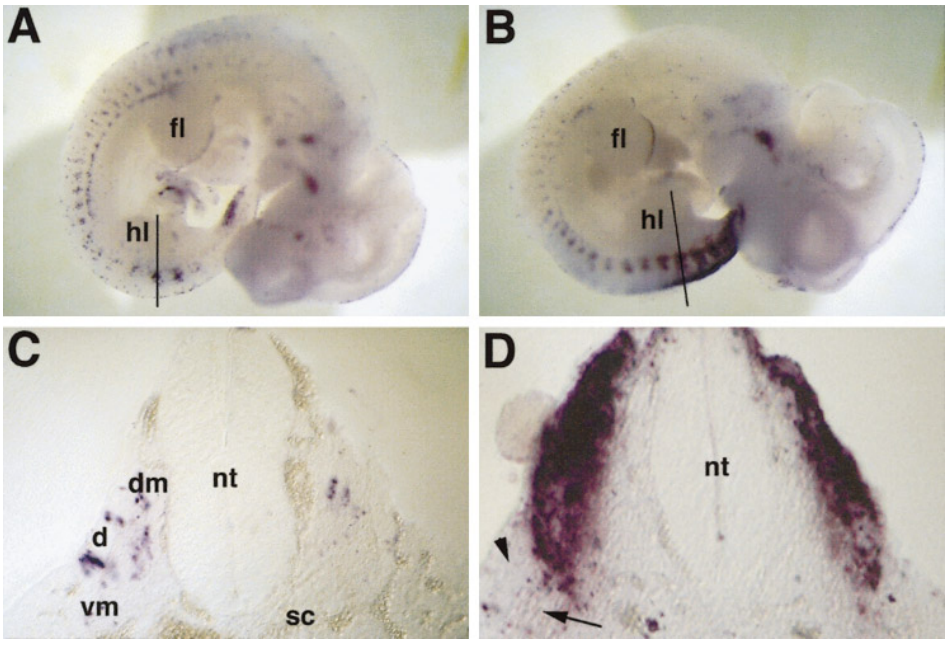

FIG. 9. Apoptotic cell death in the wild-type and *Sd* embryos. At E10.5, wild-type (A, C) and *Sd* (B, D) embryos were stained using the TUNEL technique for detecting apoptotic cell death. In the wild-type embryos, a small amount of apoptosis was detected in the caudal dermomyotome and myotomes (A, C). In the *Sd* embryos, extensive apoptosis was detected in the dorsal dermomyotome, dorsal myotome, and dorsal ectoderm, but not in the ventral dermomyotome (arrowhead), ventral myotome (arrow), neural tube, dorsal root ganglion, or sclerotome in the caudal region. Transverse sections of the embryos at caudal regions (C, D) are indicated by bars in A and B. fl, forelimb bud; hl, hindlimb bud; nt, neural tube; d, dermomyotome; dm, dorsal myotome; vm, ventral myotome.

ablation studies in avian embryos (van Straaten and Hekking, 1991; Goulding *et al.*, 1994; Dietrich *et al.*, 1997; Teillet et al, 1998). Recent observations indicate that the somite expresses *Patched* (*Ptc*), the Shh receptor (Blagden *et al.*, 1997; Marcelle *et al.*, 1997; Borycki *et al.*, 1998). Therefore, Shh might act directly to promote the survival and differentiation of the *Myf5*-expressing cells in the dorsal myotome. In this regard, we observed substantial apoptosis in the epaxial myotome and dermomyotome in the caudal region of *Sd* embryos. Since Shh has a proliferative effect on some cells (Fan *et al.*, 1995; Hirsinger *et al.*, 1997), the Shh signal might also play a role in expanding the population of lineage committed cells in the early somite, acting as a growth or survival factor. Indeed, in chicken limb bud, Shh was able to activate myogenesis in already committed myoblasts and stimulate myoblast cell proliferation (Duprez *et al.*, 1998).

The Differentiation or Maintenance of Epaxial Muscle Development Is More Sensitive to the Loss of the Ventral Axial Structures Than Hypaxial Muscle Development

The dorsomedial half of the myotome gives rise to the epaxial (back) muscle, while cells from the ventrolateral myotome differentiate to form hypaxial muscle (body wall, intercostal and limb muscles) (Christ and Ordahl, 1995). *Myf5* and *MyoD* appear to be initially expressed in a mutually exclusive manner in the dorsomedial (epaxial) and ventrolateral (hypaxial) myotomes, respectively (Smith *et al.*, 1994; Braun and Arnold, 1996; Cossu *et al.*, 1996). Our analysis of *Sd* embryos showed that epaxial muscle failed to survive and differentiate, whereas hypaxial muscle formed relatively normally in the absence of notochord and floor plate.

Other studies have shown that epaxial and hypaxial muscle development can be differentially regulated. In the neural tube mutant *open brain* (*opb*), *Myf5* gene expression is severely reduced, whereas *MyoD* expression remains normal. This result suggests that the Wnt-signaling pathway from the dorsal neural tube is necessary for the induction and differentiation of the epaxial *Myf5*-expressing cells, but not the hypaxial *MyoD*-expressing cells (Sporle *et al.*, 1996). Similarly, in *Shh* knockout mice, *Myf5* gene expression is severely affected, while *MyoD* is expressed normally, suggesting that *Shh* is also required for epaxial muscle development but not hypaxial and consistent with our data on *Sd* embryos (Chiang *et al.*, 1996). Also, while the initial analysis of *MyoD* or *Myf5* knock-out mice did not report substantial skeletal muscle abnormalities (Braun *et al.*, 1992; Rudnicki *et al.*, 1995), recent work shows that epaxial muscle development is delayed in *Myf5*-

null mice and hypaxial muscle development is delayed in *MyoD*-null mice (Kablar *et al.*, 1997). Therefore, while *MyoD* and *Myf5* can compensate for each other, the epaxial lineage appears dependent on *Myf5* and the hypaxial lineage appears dependent on *MyoD* for their normal development.

General Aspects of Notochord Function and Myogenesis

In zebrafish, Shh secreted from the ventral axial structures induces the formation of slow muscles (Blagden *et al.*, 1997). In the notochord mutants, *bozozok* (*boz*) and *no tail* (*ntl*), the adaxial muscle pioneer cells are absent and slow muscle does not form, whereas the formation of fast muscle is unaffected (Blagden *et al.*, 1997). In contrast, in the *Sd* mouse embryo, both embryonic and β-cardiac isoforms of *MHC* (Lyons *et al.*, 1990) that characterize slow muscle fibers were detected in rostral somites that develop after notochord degeneration (A.A. and S.J.T., unpublished data). Therefore, in both zebrafish and mouse, the notochord appears necessary for a subset of muscle cells, but the final characteristics of the muscle types are different.

In ascidian larva, which belong to the urochordate, the primary axial muscle lineage forms cell autonomously, whereas the secondary muscle lineage requires inductive signals. In tailless species of ascidian that do not form a notochord, the primary muscle lineage forms but the secondary muscle lineage does not (Sawalla and Jeffery, 1996). Similar to the mouse and zebrafish mutants, therefore, in ascidians one muscle lineage depends on notochord induction, whereas a second lineage does not. If the inductive mechanisms were conserved during chordate evolution, then epaxial muscle and hypaxial muscle in the mouse would be phylogenically homologous to zebrafish slow and fast muscle, or to the ascidian secondary and primary muscles.

Myogenesis in the Sd Embryo

In conclusion, the initial activation of the *Myf5* gene in the newly formed somite was independent of the notochord; however, signals from the notochord and ventral axial structures appeared necessary to achieve high levels of *Myf5* expression. In addition, signals from the notochord restricted *Myf5* expression to the dorsomedial lip of dermomyotome and expanded *Pax1* expression in the sclerotome. Finally, there was decreased differentiation and survival of the epaxial muscle cells of the dorsal myotome in somites that formed following degeneration of the notochord whereas the hypaxial muscle of the ventral myotome was not appreciably affected. These findings are consistent with other studies that implicate Shh signals from the ventral axial structures with differentiation and survival of the dorsomedial myotome (Teillet *et al.*, 1998). Further genetic and molecular analysis of epaxial and hypaxial muscle development should continue to reveal the mechanisms of myotome specification and development.

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